Evaluation of visible-light wavelengths that reduce or oxidize the plastoquinone pool in green algae with the activated F₀ rise method

H. MATTILA*, V. HAVURINNE**, T. ANTAL***, and E. TYYSTJÄRVI*

Molecular Plant Biology, Department of Life Technologies, University of Turku, 20014 Turku, Finland*
Present address: ECOMARE, CESAM, Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal**
Permanent address: Laboratory of Integrated Ecological Research, Pskov State University, 180000 Pskov, Russia***

Abstract

We recently developed a chlorophyll a fluorescence method (activated F₀ rise) for estimating if a light wavelength preferably excites PSI or PSII in plants. Here, the method was tested in green microalgae: Scenedesmus quadricauda, Scenedesmus ecornis, Scenedesmus fuscus, Chlamydomonas reinhardtii, Chlorella sorokiniana, and Ettlia oleoabundans. The Scenedesmus species displayed a plant-like action spectra of F₀ rise, suggesting that PSII/PSI absorption ratio is conserved from higher plants to green algae. F₀ rise was weak in a strain of C. reinhardtii, C. sorokiniana, and E. oleoabundans. Interestingly, another C. reinhardtii strain exhibited a strong F₀ rise. The result indicates that the same illumination can lead to different redox states of the plastoquinone pool in different algae. Flavodiiron activity enhanced the F₀ rise, presumably by oxidizing the plastoquinone pool during pre-illumination. The activity of plastid terminal oxidase, in turn, diminished the F₀ rise, but to a small degree.

Keywords: Chlamydomonas; chlorophyll fluorescence; far-red acclimation; FlvB protein; Scenedesmus.

Introduction

Nowadays, green algae are of keen interest to the scientific community due to the great potential for photosynthetic production of biofuels, hydrogen gas, carotenoids, omega-3 fatty acids, and other useful compounds (for reviews, see Stensjö et al. 2018, Bolatkhan et al. 2019, Petrova et al. 2020, Bhatia et al. 2021). Optimization of biotechnological production systems requires a deep understanding of the effects of light on rearrangements of metabolism and regulatory networks, and in particular, on the regulation of photosynthetic processes and energy balance in the algal cell. Knowledge of spectral preferences of PSI and PSII in algae can provide valuable information on the light-dependent regulation of electron fluxes through PSII and PSI, and can therefore be employed in algal biotechnology.

Highlights

- F₀ rise fluorescence can be used to probe PSII/PSI absorption ratio in green algae
- Scenedesmus quadricauda shows a similar F₀ rise spectra as higher plants
- Flavodiiron proteins contribute to the oxidation of the plastoquinone pool in white light

Abbreviations: Chl – chlorophyll; F₀ rise – post-illumination rise of chlorophyll a fluorescence; FR – far-red; flv – flavodiiron; FWHM – full width at half maximum; LL – low light; ML – measuring light; PAM – pulse amplitude modulation; PG – propyl gallate; PQ – plastoquinone; PTOX – plastid terminal oxidase; SP – saturating pulse.

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*Corresponding authors
e-mail: taras_an@mail.ru (T. Antal)
esatty@utu.fi (E. Tyystjärvi)
to optimize product yield, as previously shown, e.g., for hydrogen photoproduction in *Chlamydomonas reinhardtii* (Antal et al. 2016).

Recently, we developed a fluorescence protocol for higher plants to screen the capacity of different wavelengths of light to excite preferentially PSI or PSII and, consequently, to oxidize or reduce the thylakoid membrane plastoquinone (PQ) pool (Mattila et al. 2020). The method exploits the transient post-illumination rise of chlorophyll (Chl) $a$ fluorescence (the $F_0$ rise) that reflects reduction and subsequent oxidation of the plastoquinone (PQ) pool in the dark by stromal reductants via the machinery of cyclic electron flow (Mills et al. 1979, Endo et al. 1997, Field et al. 1998, Shikanai et al. 1998). In the activated $F_0$ rise method, a light-acclimated leaf is first illuminated with moderate actinic light for a few minutes. Then, this pre-illumination is changed to a weak monochromatic light (called activating light), and changes in Chl $a$ fluorescence yield are probed with short pulses of the measuring light of a pulse amplitude modulation (PAM) fluorometer (Mattila et al. 2020). Activating light favoring PSI excitation induces a high $F_0$ rise because oxidation of $Q_X$ is slowed down by the accumulation of PQH$_2$ and because light favoring PSII enhances the formation of $Q_X$. On the contrary, activating light exciting preferentially PSI favors the oxidation of the PQ pool while little $Q_X$ is formed, and hence the $F_0$ bump is suppressed or not observed at all. In *Arabidopsis thaliana*, the activated $F_0$ rise method is a sensitive indicator of the excitation balance between PSI and PSII; wavelengths favoring PSII reduce the plastoquinone (PQ) pool, at moderate intensity, and wavelengths favoring PSI oxidize the PQ pool (Mattila et al. 2020). The same wavelengths that cause oxidation or reduction of the PQ pool in plants also function in *C. reinhardtii*, but the variation in the redox state of the PQ pool is not as large in the alga as in the plant (Virtanen and Tyystjärvi 2022). In photosynthetic organisms harboring functional flavodiiron (Flv) proteins in the chloroplasts (Ilik et al. 2017), Flv activity is expected to affect the $F_0$ rise by depleting the stromal reductants.

In plants and green algae, the redox state of the PQ pool controls state transitions, which modify the energy balance between PSI and PSII (Allen et al. 1981, Vener et al. 1997, Finazzi et al. 2001, Depège et al. 2003, Nawrocki et al. 2016) and regulates nuclear and chloroplast gene expression (Escoubas et al. 1995, Pfannschmidt et al. 1999, Schönfeld et al. 2004). Antenna structures are known to react to the balance of the photosystems (for a review, see Lazar et al. 2022). In natural conditions with polychromatic white light, extreme oxidation or reduction of the PQ pool is unlikely, as electron transfer reactions of both photosystems function. Therefore, methods to probe the redox state of the PQ pool without setting a strong bias toward either extreme (with the help of sudden high light, far-red light, or inhibitors of electron transfer) are needed to understand the PQ-based regulation in vivo.

The knowledge obtained from higher plants cannot be directly used with algae, because the outer light-harvesting complexes of PSII and PSI of the green alga *C. reinhardtii*, for example, have been shown to greatly differ from those of plants (Tokutsu et al. 2012, Kawakami et al. 2019, Kubota-Kawai et al. 2019). Further differences may exist in other algae, and therefore methods are needed for the determination of wavelengths preferentially exciting one photosystem over the other in algae. In addition, the reactions involved in transient alterations of the PQ redox state during the post-illumination period may differ between green algae and plants due to differences in cyclic and pseudo-cyclic electron flow pathways and because of more substantial chlororespiration in green algae than in plants (Antal et al. 2013; for reviews, see Alric 2010, Alric and Johnson 2017). These factors can influence the $F_0$ rise pattern, raising the question about possible limitations and applicability of the activated $F_0$ rise method in microalgae.

In the current work, we tested the $F_0$ rise protocol in six algal species. Microalgae from the genera *Scenedesmus, Chlamydomonas, Chlorella,* and *Ettlia* were chosen for the current study, as these algae are widely used as model organisms and in studies on the production of biofuels and molecular hydrogen. We found that *Scenedesmus* strains show explicit $F_0$ rise with a plant-like action spectrum, whereas *C. reinhardtii, Chlorella sorokiniana,* and *Ettlia oleobundans* exhibited a reduced $F_0$ rise. In addition, the results indicate that flavodiiron activity greatly affects the redox state of the photosynthetic electron transfer chain during illumination, while the plastid terminal oxidase (PTOX) may play a minor role during the post-illumination period.

**Materials and methods**

**Strains and growth conditions:** *C. sorokiniana* (Shihira & R.W. Krauss) and three strains of *Scenedesmus* *(S. quadricauda* (Turpin) Brébisson, *S. ecoris* (Ehrenberg) Chodat, and *S. fuscus* (Kirchner) E. Hegewald) were ordered from CCALA (http://ccala.butbn.cas.cz). Three *C. reinhardtii* strains, 125 (137C), the FlvB deletion strain 242 208, and its wild type cc4533, were obtained from the Chlamydomonas center (http://www.chlamycollection.org). The flvB mutant is further described by Jokel et al. (2018). The oleaginous species *E. oleobundans* (earlier known as *Neochloris oleobundans*) was ordered from *UTEX* (1185) (http://utex.org).

All algal cultures were grown autotrophically in BG11 medium (Rippka et al. 1979) buffered to pH 7.5 with 20 mM Hepes–KOH, except for *C. reinhardtii*, which was cultivated autotrophically in high salt medium (HSM) (Sueoka 1960). All cultures were started with an OD$_{730}$ = 0.1. Three experimental conditions, marked as A, B, and C, were used for the cultivation of the algae:

- **Condition A.** Semi-continuous cultivation in 1-L flasks with constant bubbling by air and stirring at 26°C under illumination from a fluorescent lamp at photosynthetic photon flux density (PPFD) of 140 μmol m$^{-2}$ s$^{-1}$. The spectral distribution of the light is shown in Fig. 1S (supplement).
- **Condition C.** Directly used with algae, because the outer light-harvesting complexes of PSII and PSI of the green alga *C. reinhardtii*,...
with fresh medium to 0.35. Due to the daily growth, OD$_{730}$ fluctuated between 0.35 and 0.40. The measurements were done after 7 d of cultivation.

- **Condition B.** Batch cultivation in 100-ml Erlenmeyer flasks on a shaker (100 rpm) at 26°C under a white LED matrix (Fig. 1S) at PPFD of 200 µmol m$^{-2}$ s$^{-1}$ in an Algaenator (PSI, Czech Republic). The measurements were done after 7 d of cultivation after which OD$_{730}$ had reached approximately 0.6.
- **Condition C.** Batch cultivation in 100-ml Erlenmeyer flasks on a shaker (100 rpm) at 26°C under a fluorescent lamp (Fig. 1S) at PPFD of 35 µmol m$^{-2}$ s$^{-1}$. The measurements were done after 14 d of cultivation after which OD$_{730}$ had reached approximately 0.8.

PPFD was measured with a wavelength-calibrated quantum sensor (LiCor, Lincoln, NE) and light spectra were measured with an STS-VIS spectrometer (Ocean Insight, USA).

**F$_0$ rise measurements – activated F$_0$ rise:** Chl $a$ fluorescence was measured with a PAM-101 fluorometer (Heinz Walz GmbH, Germany) according to the activated F$_0$ rise protocol described by Mattila et al. (2020).

An algal culture was pre-incubated for 1 h in weak white light (PPFD of 5–10 µmol m$^{-2}$ s$^{-1}$) at room temperature. Algae were then collected on a glass fiber filter (with a diameter of 2.4 cm; VWR) with a final Chl concentration of 70–75 mg m$^{-2}$; Chl was extracted by at least 24-h incubation in methanol at 4°C in the dark (InskEEP and Bloom 1985) from separate cultures with known OD to estimate the needed algal volume. Then, a weak red measuring light (ML) was switched on using the OMNICOR illuminator, equipped with a halogen bulb, to set the OD to 0.6 s on/5 s off. After 20 s, a saturating pulse (SP) (PPFD of 5,000 µmol m$^{-2}$ s$^{-1}$, 0.8 s) was fired and an actinic light (PPFD of 50 µmol m$^{-2}$ s$^{-1}$) was switched on simultaneously, as shown in Fig. 2S (supplement).

A Walz KL-1500 illuminator, equipped with a halogen bulb was used as a light source for this pre-illumination (for the spectra, see Fig. 1S) unless otherwise specified. After 180 s of the pre-illumination, a second SP was fired to estimate the magnitude of nonphotochemical quenching (NPQ), which can interfere with the F$_0$ rise measurements. After a further 60 s of illumination, the pre-illumination and ML were switched off and a monochromatic activating light (PPFD of 2.5 µmol m$^{-2}$ s$^{-1}$) defined with a 10-nm full width at half maximum (FWHM) line filter (Corion, Newport Corp., Irvine, CA), was switched on. From here on, the ML was used in a chopped mode using cycles of 0.6 s on/5 s off unless otherwise mentioned. Activating light of 660 or 700 nm (FWHM 10 nm; Corion) that functions as PSII or PSI light, respectively, in A. thaliana (Mattila et al. 2020), was used to test whether a particular algal species can exert the post-illumination fluorescence rise. Activated F$_0$ rise was quantified by integrating the fluorescence signal during 60 s of the post-illumination period (fluorescence values lower than those at the moment when the actinic illumination ended, were subtracted).

**F$_0$ rise measurements – traditional F$_0$ rise:** In some cases, as indicated, F$_0$ rise was measured with the ‘original’ protocol, with a Multi-Color PAM (MC-PAM; Heinz Walz GmbH). Blue (480 nm, PPFD ~2.5 µmol m$^{-2}$ s$^{-1}$) ML of MC-PAM functioned as the activating light, and was on for the whole measurement period, with the frequency of 5 kHz (before and after the pre-illumination) or 20 kHz (during the pre-illumination). White actinic light (PPFD of 50 µmol m$^{-2}$ s$^{-1}$) or far-red light (for the spectra, see Fig. 1S) of the MC-PAM was used as the pre-illumination, as indicated. Activated F$_0$ rise was quantified by integrating the fluorescence signal during 100 s of the post-illumination period. In one set of experiments, 1 mM propyl gallate, an inhibitor of PTOX, was added to the (liquid) algal culture 5 min before the fluorescence measurement started. Otherwise, the conditions were identical to those of the activated F$_0$ rise protocol described above.

**P$_{700}$ measurements:** Simultaneous measurements of fluorescence and P$_{700}$ absorbance signal were conducted with Dual KLAS NIR, with the ED-101US/MD accessory cuvette for liquid measurements (Heinz Walz GmbH). Since no calibration was available for green algae, only the P$_{700}$ signal and fluorescence were measured. The cuvette temperature was set to 26°C, blue ML was used and far-red was set to maximum. After control measurements, oxygen was removed by adding 6 mM glucose, 800 U ml$^{-1}$ glucose oxidase, and 8 U ml$^{-1}$ catalase (Sigma Aldrich), and the sample was incubated for 15 min at room temperature in the dark after which the measurement was repeated.

**Statistics:** Statistical differences were tested by calculating the Student's t-test (heteroscedastic) in Microsoft Excel, based on at least three independent replicates. Asterisks ***, **, and * indicate a probability of P<0.001, P<0.01, and P<0.05, respectively, of the null hypothesis.

**Results**

Application of the F$_0$ rise protocol to green microalgae:
We recorded the post-illumination fluorescence rise (F$_0$ rise) using 660 and 700-nm weak activating light for preferential excitation of PSII or PSI, respectively (for the protocol, see Fig. 2S). The method was originally designed to screen the photosystem preference of a visible light wavelength in higher plants (Mattila et al. 2020). Here, preliminary measurements in six green algal species revealed that three Scenedesmus species, namely *S. quadricauda*, *S. ecornis*, and *S. fuscus*, showed a rise in F$_0$ fluorescence level under 660-nm activating light and, like plants, lacked an F$_0$ rise at 700-nm activating light (Table 1). Examples of the fluorescence traces from *S. quadricauda* are shown in Fig. 1; switching off the white pre-illumination and switching on the weak 660-nm activating light caused a sharp, ~70% increase in the fluorescence level during the first 5 s, followed by a slow decline to the initial fluorescence level. When activating light of 700 nm was applied, no post-illumination fluorescence rise was observed.

*S. oleoabundans*, in turn, showed almost no F$_0$ rise in response to 660 nm, nor to 700 nm activating light. *C. reinhardtii* and *C. sorokiniana* exhibited differences
Table 1. Occurrence of the F₀ rise in different green algae species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strains</th>
<th>Protocol</th>
<th>F₀ rise pattern</th>
<th>Cultivation conditions</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scenedesmus quadricauda</td>
<td></td>
<td>660-nm activating light, PAM-101</td>
<td>Plant-like</td>
<td>A, B, C</td>
<td>BG11</td>
</tr>
<tr>
<td>Scenedesmus ecornis</td>
<td></td>
<td>660-nm activating light, PAM-101</td>
<td>Plant-like</td>
<td>C</td>
<td>BG11</td>
</tr>
<tr>
<td>Scenedesmus fuscus</td>
<td></td>
<td>660-nm activating light, PAM-101</td>
<td>Plant-like</td>
<td>C</td>
<td>BG11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>480-nm ML, MC-PAM</td>
<td>Plant-like</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Chlorella sorokiniana</td>
<td>125 (137C) cc4533</td>
<td>660-nm activating light, PAM-101</td>
<td>Unobvious</td>
<td>A</td>
<td>BG11</td>
</tr>
<tr>
<td>Chlamydomonas reinhardtii</td>
<td>cc4533</td>
<td>480-nm ML, MC-PAM</td>
<td>Unobvious</td>
<td>A</td>
<td>HSM</td>
</tr>
<tr>
<td></td>
<td>flvB (cc4533)</td>
<td>480-nm ML, MC-PAM</td>
<td>Unobvious</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Ettlia oleoabundans</td>
<td></td>
<td>660-nm activating light, PAM-101</td>
<td>Unobvious</td>
<td>A</td>
<td>BG11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>480-nm ML, MC-PAM</td>
<td>Unobvious</td>
<td>B</td>
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</tbody>
</table>

Fig. 1. Chlorophyll a fluorescence traces measured from Scenedesmus quadricauda, grown under conditions A (semi-continuous, moderate light). White light from a KL-1500 illuminator was used as the pre-illumination (PPFD of 50 µmol m⁻² s⁻¹). The wavelengths of the monochromatic activating light (PPFD of 2.5 µmol m⁻² s⁻¹) were 660 nm (grey trace) and 700 nm (black trace). The 700-nm curve has been shifted upwards and to the right for clarity. Representative curves are shown.

between activated F₀ rise traces measured with 660 and 700-nm activating light, although a clear transient increase in the fluorescence yield after the switch-off of the pre-illumination was absent (Table 1).

Exposure of S. quadricauda cells to the white pre-illumination (PPFD of 50 µmol m⁻² s⁻¹) for 3 min induced moderate NPQ as indicated by the finding that the Fₐ′ level was about 20% lower than Fₐ (Fig. 1). This level of NPQ, even if it partially relaxes during the monitoring of the F₀ rise, would only have a minor influence on the F₀ rise transient.

**Action spectra of activated F₀ rise in S. quadricauda and S. fuscus:** Since S. quadricauda expressed a clear F₀ rise pattern, we used this species to measure the action spectra of the F₀ rise. S. quadricauda was grown under three different growth conditions (semi-continuous growth under moderate light, and batch growth under high or low light, indicated by the letters A, B, and C, respectively; see ‘Materials and methods’ for details). S. quadricauda showed a high F₀ rise at activating light wavelengths of 470–490 nm (blue peak), 650–660 nm (red peak), and 560–600 nm (broad green peak), whereas 420–450 nm (blue deep), 520–530 nm (green deep), 620–630 nm (orange deep), and 680–690 nm (red deep) activating light exhibited a low capacity to generate an F₀ rise (Fig. 2A–C). Such features are also observed in an F₀ spectrum measured from tobacco leaves (Mattila et al. 2020). The amplitude of the F₀ rise was higher in the alga than that in the plant. However, optical differences between plant leaves and algal cells may have contributed to the difference. Furthermore, the action spectra were similar in S. quadricauda grown under the three different growth conditions; growth conditions appeared to mostly affect the shape and maximum values of the broad green peak (560–600 nm), which did not have a consistent maximum in the three conditions. The maximal amplitudes of the activated F₀ rise were also higher in cells grown under high light (condition B) and the red peak (660–670 nm) was shifted to the right, compared to the other conditions. The LED illumination used in condition B (see Fig. 1S for the spectra) contained more red and far-red light than the fluorescent light used for other growth conditions.

The action spectrum of the F₀ rise was also measured from S. fuscus (grown under condition C), with a lower resolution. The spectrum had the same peaks and deeps as that of S. quadricauda (Fig. 2D).

**Effect of PTOX activity on F₀ rise:** To get more insights into the fact that not all algal species showed an F₀ rise (Table 1), we repeated the F₀ rise measurement in the presence of propyl gallate, a PTOX inhibitor, with S. fuscus (an alga showing a clear F₀ rise) and with E. oleoabundans (an alga showing no F₀ rise). In this case, we used the ‘original’ F₀ rise protocol, where the weak measuring light of the fluorometer (480 nm in this case) is continuously on during the F₀ rise measurement and acts as the activating light. S. fuscus showed again a clear F₀ rise, but also E. oleoabundans showed a tiny F₀ rise (Fig. 3). The addition of propyl gallate increased the size of the F₀ rise in both algae, even though E. oleoabundans still showed a very small F₀ rise (Fig. 3). The addition of propyl gallate
increased also the amount of NPQ, especially in *S. fuscus*, and therefore, the measurements were also performed with a dimmer pre-illumination (PPFD of 35 µmol m⁻² s⁻¹) for *S. fuscus* and with a far-red light (see Fig. 1S for the light spectrum) as the pre-illumination for both *S. fuscus* and *E. oleoabundans*. Lowering the pre-illumination intensity reduced NPQ formation (Fig. 3A), and after the far-red pre-illumination, no NPQ developed (Fig. 3A,B). Far-red pre-illumination increased the size of the F₀ rise in both algae, but the increase was significant only in *E. oleoabundans*. The addition of propyl gallate further increased the size of the F₀ rise only in *E. oleoabundans* (Fig. 3). In addition, in the case of *S. fuscus* but not of *E. oleoabundans*, propyl gallate addition slowed down the F₀ fluorescence decrease after the F₀ rise, in all the treatments (Fig. 3A,B).

**F₀ rise in *C. reinhardtii***: As indicated in Table 1, *C. reinhardtii* [the 125 (137C) wild type] did not show a clear F₀ rise. However, fluorescence traces measured with 660 and 700 nm activating light showed obvious differences; with 660 nm activating light, the fluorescence yield declined gradually to a stable minimal level in 20 s while the decrease of fluorescence yield was rapid when 700 nm activating light was used (Fig. 4A).

The fluorescence traces of *C. reinhardtii* resembled those measured from *Arabidopsis*, when using a pre-illumination that reduces rather than oxidizes the PQ pool (Mattila et al. 2020). Therefore, the measurement protocol was modified so that 690 nm light (PPFD of 50 µmol m⁻² s⁻¹) was used as the pre-illumination for efficient oxidation of the PQ pool. Furthermore, blue wavelengths of 420 nm (PSI light) and 470 nm (PSII light) were used as activating lights because 470 nm actinic light induced a slightly stronger activated F₀ rise in *S. quadricauda* than that of 660 nm activating light (Fig. 2). The ML cycles for estimating F₀ rise were also applied at a higher frequency (0.6 s on/1 s off). Application of 420 nm PSI light as the activating light caused a rapid (1 s) drop in fluorescence yield after switching off the 690 nm pre-illumination, and the fluorescence level remained unaltered until the end of the measurement (Fig. 4B). When 470 nm activating light (PSII light) was applied, fluorescence initially declined for 1 s, and thereafter increased for 5 s, and then gradually decreased to a minimum (Fig. 4B). It seems, therefore, that the white light from *KL-1500* indeed did not oxidize the PQ pool in *C. reinhardtii* 125 (137C). The F₀ rise after the 690 nm pre-illumination was still quite small.

To further understand the conditions determining the size of the F₀ rise in *C. reinhardtii*, we used a mutant lacking the FlvB protein (Jokel et al. 2018) and its corresponding wild type [cc4533, a different strain from the above used *C. reinhardtii* 125 (137C)]. Here we used the white or far-red light of the MC-PAM fluorometer, as indicated, as the pre-illumination light while the weak 480 nm measuring light was used as the activating light. In contrast to the *C. reinhardtii* 125 (137C), the cc4533 strain produced a strong F₀ rise, under both types of the pre-illumination light (Fig. 5). The deletion of the FlvB, in turn, clearly diminished the size of the F₀ rise (Fig. 5).

**Effect of oxygen removal on P₇₀₀ kinetics in *S. fuscus* and *E. oleoabundans***: To test if flavodiiron activity could explain the varying sizes of the F₀ rise also in the other green algal species, we measured the P₇₀₀ (the primary electron donor of PSI) oxidation capacity of *S. fuscus* and *E. oleoabundans* in the presence and absence of oxygen. In the case of *S. fuscus*, far-red and high-light pulses were able to reduce P₇₀₀ in the presence of oxygen, but when oxygen was removed, only a transient spike upon the high-light pulse was observed (Fig. 6A,B). P₇₀₀ oxidation pattern in *E. oleoabundans*, in turn, was not affected by oxygen removal (Fig. 6C,D).
Discussion

In higher plants, the wavelengths causing a high $F_0$ rise were shown to favor PSII over PSI, and also to reduce the PQ pool at a moderate intensity. A high activated $F_0$ rise was obtained with 460–500 nm (blue peak), 560 nm (green peak), and 650–660 nm (red peak) activating light, whereas the $F_0$ rise was weak or missing at 420–450 nm (blue deep), 520 nm (green deep), 630 nm (orange deep), and 680–690 nm (red deep) lights. It was concluded that the form of the action spectrum of the $F_0$ rise is mainly determined by the ratio of Chl b to Chl a in the antenna complexes (Mattila et al. 2020). In the present study, a similar $F_0$ rise protocol was applied to several green algal species but only Scenedesmus species ($S$. quadricauda, $S$. ecornis, $S$. fuscus) exhibited an explicit plant-like $F_0$ rise. The $F_0$ rise spectra of $S$. quadricauda (Fig. 2) were highly similar to that of tobacco (Mattila et al. 2020), suggesting that the spectral properties of the two photosystems are very conserved between land plants and green algae (Viridiplantae), even though algae are characterized by peculiar structural organizations of peripheral antenna complexes (for a review, see Rochaix 2014). Also, the redox state of the PQ pool, after illumination with several wavelengths favoring either PSII or PSI, shows a similar response in Arabidopsis and C. reinhardtii (Mattila et al. 2020, Virtanen and Tyystjärvi 2022). However, more green algal species should be investigated.

The action spectra of the $F_0$ rise of $S$. quadricauda were very similar after cultivation of the algae in three different experimental conditions differing in light quantity and quality (Figs. 1S, 2S). Interestingly, though, 670–680 nm light produced a relatively high $F_0$ rise only when $S$. quadricauda was grown under conditions B, where the growth light had significant red and far-red contribu-
tions, unlike in the other growth conditions. This might suggest that the growth under red and far-red enriched light modified absorption properties of the antennae in *S. quadricauda*, possibly due to state transitions and/or other modifications of antennae composition and structure (Ueno *et al.* 2019, Wolf and Blankenship 2019). The observation that higher values of F₀ rise were observed after growth under high light (condition B; Fig. 2) may suggest a better capacity of these cells to keep the PQ pool oxidized during the pre-illumination.

In plant leaves, the F₀ rise method can be used, at most wavelengths, to predict the capacity of the wavelength to reduce/oxidize the PQ pool (Mattila *et al.* 2020). However, the 630 nm orange deep (i.e., a PSI light) is an exception, as the *A. thaliana* PQ pool was found to remain ~50% reduced at this wavelength. The finding that the activated F₀ rise shows a clear orange deep also in *S. quadricauda* (Fig. 2) suggests that 630 nm light truly favors PSI.

Unlike the *Scenedesmus* species, *C. reinhardtii* [the 125 (137C) strain], *C. sorokiniana*, and *E. oleoabundans* did not exhibit a clear F₀ rise (with a PSII-activating light) when white pre-illumination was used (Table 1; Figs. 3, 4). The size of the F₀ rise can be modulated by four factors: (1) the oxidation state of the PQ pool at the end of the pre-illumination, as it was shown that a big F₀ bump only occurs if the pre-illumination oxidizes the PQ pool (Mattila *et al.* 2020), (2) the PSII/PSI nature of the activating light, (3) the rate of the reduction of the PQ pool after switching off the pre-illumination, which in *A. thaliana* was shown to occur via the activity of the chloroplast NADH dehydrogenase-like (NDH) complex (Mattila *et al.* 2020), and (4), theoretically, by the rate of the oxidation of the PQ pool after switching off the pre-illumination. A clear, although small F₀ rise could then be seen in *C. reinhardtii* [the 125 (137C) strain] and *E. oleoabundans* by using far-red light (a clear PSI light) as the pre-illumination.

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**Fig. 5.** Fluorescence traces (*A*) and quantification of the F₀ rise (*B*) in *Chlamydomonas reinhardtii* (cc4533) wild type (wt), and FlvB deletion mutant (flv 208), grown under conditions B (batch, high light). White (WL) or far-red (FR) light from an MC-PAM was used as the pre-illumination (PPFD of 50 µmol m⁻² s⁻¹), as indicated. A 480-nm measuring light (PPFD ~2.5 µmol m⁻² s⁻¹) was used as the activating light. The fluorescence traces in (*A*) have been shifted for clarity. Representative curves are shown. Data in (*B*) represent the mean of three replicates and the standard error. The asterisks indicate statistical significance between the indicated groups.

**Fig. 6.** Fluorescence and P₇₀₀ traces in *Scenedesmus fuscus* (*A,B*) and *Ettlia oleoabundans* (*C,D*), during aerobic (*A,C*) and anaerobic (*B,D*) conditions. FR indicates a far-red light and SP a saturating pulse. The algae were grown under conditions B (batch, high light). Representative curves are shown.

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(Figs. 3, 4), indicating that the white light illumination with a KL-1500 illuminator did not oxidize the PQ pool in these algae. In contrast, in A. thalaina, the KL-1500 tends to oxidize the PQ pool (Mattila et al. 2020), as well as presumably also in the Scenedesmus species used here.

In the other C. reinhardtii strain (cc4533), flavodiiron proteins seemed to contribute to the oxidation of the PQ pool during white light pre-illumination, as an absence of the FvB and FvA proteins (Jokel et al. 2018) decreased the amplitude of the F₀ rise (Fig. 5). The explanation is supported by the observation that in the flvB mutant fluorescence yield stayed at a higher level during the pre-illumination, compared to the wild type (Fig. 5A). Large effects of the flavodiiron proteins on chlorophyll fluorescence phenomena in C. reinhardtii have been demonstrated also earlier (Jokel et al. 2018). Intriguingly, the two C. reinhardtii strains used showed very different F₀ rise patterns (Figs. 4, 5), suggesting different strategies for balancing the electron transfer chain. However, we cannot rule out the possibility that different growth conditions or F₀ protocols could have contributed to the differences.

Whether flavodiiron proteins are important also in other algae in keeping the PQ pool oxidized during a white light illumination is difficult to judge as the presence of flavodiiron proteins in the studied species (except for C. reinhardtii) is not known. Scenedesmus shows light-dependent oxygen reduction (Radmer and Kok 1976), which has been interpreted to indicate the presence of flavodiiron proteins (e.g., Pelletier et al. 2010), but BLAST searches for genes coding for proteins related to Chlamydomonas FlvA or FlvB proteins did not yield significant homologs in Scenedesmus species. BLAST searches with the same proteins did reveal significant homologs in Chlorella ohadii and Chlorella variabilis, indicating that flavodiiron proteins can also be found in Trebouxiophyceae (incl. Chlorella). As flavodiiron proteins donate electrons to oxygen, the sensitivity of P₅₀₀ kinetics to anaerobiosis has been previously used to screen flavodiiron-like activity in various algal species (Shimakawa et al. 2019). P₅₀₀ oxidation kinetics in E. oleoabundans (no or very small F₀ rise) were unaffected by the removal of oxygen, in contrast to S. fuscus (big F₀ rise) where P₅₀₀ oxidation was disturbed in the absence of oxygen (Fig. 6). It can be hypothesized, then, that in those species with a high F₀ rise, the PQ pool remained at a more oxidized state during a white light pre-illumination, due to a high flavodiiron activity.

Even with the far-red pre-illumination the F₀ rise was very small in the 125 (137C) strain of C. reinhardtii and in E. oleoabundans (Figs. 3, 4), suggesting that the difficulties to keep the PQ pool oxidized during a white pre-illumination were not the only reason for a small F₀ rise, but either the post-illumination reduction of the PQ pool was slow, or its concomitant re-oxidation was fast. PTOX is the only known mechanism that oxidizes the (thylakoid) PQ pool in the dark. Indeed, the ptx2 mutant of C. reinhardtii has earlier been shown to possess a high F₀ rise (Houille-Vernes et al. 2011). The addition of propyl gallate (a PTOX inhibitor) did increase the size of the F₀ rise in E. oleoabundans and S. fuscus, and also

the disappearance of the F₀ bump was hindered in S. fuscus (Fig. 3). However, the changes were small, suggesting that the rate of the PQ oxidation by PTOX was slow in the studied algae.

In C. reinhardtii cc4533 and E. oleoabundans, the F₀ rise level after the F₀ bump decreased slower after far-red pre-illumination than after white pre-illumination (Figs. 3, 5). Changes in NPQ relaxation or the rate of PQ reduction in the dark may explain these observations, rather than differences in PTOX activity, as the same trend was also observed with propyl gallate in E. oleoabundans (Fig. 3B).

In green algae and plants, the PGR5/PGLRL1 and NDH-1 or NDH-2 mediated pathways are known contributors to the nonphotochemical (dark) reduction of the PQ pool (for a review, see Pelletier et al. 2016), and consequently to the F₀ rise. Electron transfer through the PGR5/PGLRL1-dependent route is only weakly associated with the F₀ rise in plants (Munekage et al. 2002, Nellapalli et al. 2015) whereas F₀ rise is suppressed in the NDH-1 deficient ndh-0 mutant of A. thalaina (Shikanai et al. 1998, Mattila et al. 2020). Algae belonging to classes Chlorophyceae (Chlamydomonas, Scenedesmus, Ettlia) and Trebouxiophyceae (Chlorella) have lost chloroplastic ndh genes (Pelletier et al. 2016). Nevertheless, in C. reinhardtii, the nucleus-encoded Nda2, a type II NAD(P)H dehydrogenase (NDH-2), mediates electron transfer from NAD(P)H to PQ (Desplats et al. 2009). The presence of an F₀ rise in C. reinhardtii (Figs. 4, 5) suggests that NDH-2 activity, too, can support an F₀ rise phenomenon. A BLAST search for sequences producing significant alignments with Nda2 of C. reinhardtii (ED996450.1) revealed five proteins in Chlorella variabilis with 32, 41, 45, 49, and 58% similarity but no significant similarity was found in Tetradesmus obliquus (former Scenedesmus obliquus). Thus, the presence of NDH-2 (and consequently the mechanism of F₀ rise formation) in the species used in the present study, except for C. reinhardtii, is not known. Anyway, the presence of an F₀ rise in the studied Scenedesmus, Chlorella, and Tetradesmus species suggests that these species, too, have a pathway by which ferredoxin or NADPH reduces the PQ pool.

The dynamics of the stromal electron donors during the post-illumination period may also explain differences in sizes and patterns of the F₀ rise between different algal species or different growth conditions. Reduction of NADP⁺ depends on reduced ferredoxin, and therefore rapid oxidation of NADPH either by the remaining activity of the Calvin–Benson cycle, stromal flavodiiron proteins, or Mehler’s reaction would lower the F₀ rise irrespective of whether reduction of PQ depends on ferredoxin or NADPH as the electron donor. It is also possible that PSI and PSII antennas in those algae showing a small F₀ rise have relatively similar absorption properties, and therefore visible light wavelengths do not as clearly favor one photosystem over the other.

In conclusion, the findings of our work show that the spectral distribution of PSII and PSI excitation can be very similar between land plants and green algae. However,
we showed that the magnitude and duration of the F₀ rise vary significantly among algae species. This diversity suggests variation in the balance between reduction and oxidation of the PQ pool during both the pre-illumination and the post-illumination period; flavodiiron proteins appear to be important during illumination while PTOX has a minor role after the light has been switched off. The F₀ rise method can be employed for screening alternative electron transport pathways in green algae species as well as in mutants. However, such implementation requires further exploration of the mechanisms involved in the formation of the F₀ rise in green algae.

References


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